

IN THE SPECIFICATION:

Please amend paragraphs [0036] and [0037] as follows:

[0036] FIG. 21A is a direct neuraminidase assay that was performed on recombinant EPO produced in ~~PER.C6-~~ PER.C6™ cells (human embryonic retinoblast cell line containing in its genome human adenovirus type 5 (Ad5) E1A and E1B coding sequences (nt. 459-3510) under the control of the human phosphoglycerate kinase (PGK) promoter) after transfection with EPO expression vectors.

[0037] FIG. 21B is a FACS analysis using FITC-labeled anti-DIG antibody directed to CHO cells and ~~PER.C6-~~ PER.C6™ cells.

Please amend paragraphs [0040] and [0041] as follows:

[0040] FIG. 23 is graph showing the activity of two samples derived from ~~PER.C6/E2A~~ PER.C6™/E2A cells that were transfected with an EPO expression vector.

[0041] FIG. 24 is a Western blot of transient UBS-54 expression in ~~PER.C6~~ PER.C6™ cells using antibody directed to human(ized) IgG1 sub-units.

Please amend paragraphs [0043] and [0044] as follows:

[0043] In order to achieve large-scale (continuous) production of recombinant proteins through cell culture, it is preferred in the art to have cells capable of growing without the necessity of anchorage. The cells of the present invention have that capability. The anchorage-independent growth capability is improved when the cells include a sequence encoding E2A or a functional derivative or analogue or fragment thereof in its genome, wherein preferably the E2A encoding sequence encodes a temperature sensitive mutant E2A, such as ts125. To have a clean, safe production system from which it is easy to isolate the desired recombinant protein, it is preferred to have a method according to the invention, wherein the human cell includes no other adenoviral sequences. The most preferred cell for the methods and uses of the invention is ~~PER.C6-~~ PER.C6™ as deposited under ECACC no. 96022940 with the Centre for Applied

Microbiology and Research Authority (European Collection of Animal Cell Cultures), Porton Down, Salisbury, Wiltshire SP4, OJG, United Kingdom, an International Depository Authority, on February 29, 1996 or a derivative thereof. PER.C6 PER.C6™ behaves better in handling than, for instance, transformed human 293 cells that have also been immortalized by the E1 region from adenovirus. PER.C6 PER.C6™ cells have been characterized and have been documented very extensively because they behave significantly better in the process of upscaling, suspension growth and growth factor independence. Especially the fact that PER.C6 PER.C6™ can be brought in suspension in a highly reproducible manner is something that makes it very suitable for large-scale production. Furthermore, the PER.C6 PER.C6™ cell line has been characterized for bioreactor growth in which it grows to very high densities.

[0044] The cells according to the invention, in particular PER.C6 PER.C6™, have the additional advantage that they can be cultured in the absence of animal- or human-derived serum or animal- or human-derived serum components. Thus isolation is easier, while the safety is enhanced due to the absence of additional human or animal proteins in the culture, and the system is very reliable (synthetic media are the best in reproducibility). Furthermore, the presence of the Early region 1A ("E1A") of adenovirus adds another level of advantages as compared to (human) cell lines that lack this particular gene. E1A as a transcriptional activator is known to enhance transcription from the enhancer/promoter of the CMV Immediate Early genes (Olive et al., 1990, Gorman et al., 1989). When the recombinant protein to be produced is under the control of the CMV enhancer/promoter, expression levels increase in the cells and not in cells that lack E1A. The invention therefore further provides a method for enhancing production of a recombinant proteinaceous substance in a eukaryotic cell, including providing the eukaryotic cell with a nucleic acid encoding at least part of the proteinaceous substance, wherein the coding sequence is under control of a CMV-promoter, an E1A promoter or a functional homologue, derivative and/or fragment of either and further providing the cell with E1A activity or E1A-like activity. Like the CMV promoter, E1A promoters are more active in cells expressing one or more E1A products than in cells not expressing such products. It is known that indeed the E1A expression enhancement is a characteristic of several other promoters. For the present invention, such promoters are considered to be functional homologues of E1A promoters. The E1A effect can be mediated through the attraction of transcription activators, the E1A promoter or

homologue thereof, and/or through the removal/avoiding attachment of transcriptional repressors to the promoter. The binding of activators and repressors to a promoter occurs in a sequence-dependent fashion. A functional derivative and or fragment of an E1A promoter or homologue thereof therefore at least includes the nucleic acid binding sequence of at least one E1A protein regulated activator and/or repressor.

Please amend paragraph [0046] as follows:

[0046] The invention further provides the use of a human cell for the production of a human recombinant protein, the cell having a sequence encoding at least an immortalising E1 protein of an adenovirus or a functional derivative, homologue or fragment thereof in its genome, which cell does not produce structural adenoviral proteins. In another embodiment, the invention provides such a use wherein the human cell is derived from a primary cell, preferably wherein the human cell is a PER.C6-PER.C6TM cell or a derivative thereof.

Please amend paragraphs [0049] and [0050] as follows:

[0049] In another embodiment, the invention provides a human cell having a sequence encoding E1 of an adenovirus or a functional derivative, homologue or fragment thereof in its genome, which cell does not produce structural adenoviral proteins, and having a gene encoding a human recombinant protein, preferably a human cell which is derived from PER.C6-PER.C6TM as deposited under ECACC no. 96022940.

[0050] In yet another embodiment, the invention provides such a human cell, PER.C6-PER.C6TM/E2A, which further includes a sequence encoding E2A or a functional derivative or analogue or fragment thereof in its genome, preferably wherein the E2A is temperature sensitive.

Please amend paragraphs [0052] and [0053] as follows:

[0052] The present invention also includes a novel human immortalized cell line for this purpose and the uses thereof for production. PER.C6-PER.C6TM cells (PCT International Patent Publication WO 97/00326 or U.S. Patent 5,994,128) were generated by transfection of primary human embryonic retina cells using a plasmid that contained the adenovirus serotype 5

(Ad5) E1A- and E1B-coding sequences (Ad5 nucleotides 459-3510 (SEQ ID NO: 33)) under the control of the human phosphoglycerate kinase ("PGK") promoter.

[0053] The following features make PER.C6-PER.C6TM particularly useful as a host for recombinant protein production: 1. fully characterized human cell line; 2. developed in compliance with GLP; 3. can be grown as suspension cultures in defined serum-free medium devoid of any human- or animal-derived proteins; 4. growth compatible with roller bottles, shaker flasks, spinner flasks and bioreactors with doubling times of about 35 hrs; 5. presence of E1A causing an up-regulation of expression of genes that are under the control of the CMV enhancer/promoter; 6. presence of E1B which prevents p53-dependent apoptosis possibly enhanced through overexpression of the recombinant transgene.

Please amend paragraphs [0076] through [0078] as follows:

[0076] To have a clean and safe production system from which it is easy to isolate the desired immunoglobulins, it is preferred to have a method according to the invention, wherein the human cell includes no other adenoviral sequences. The most preferred cell for the methods and uses of the invention is PER.C6-PER.C6TM or a derivative thereof as deposited under ECACC no. 96022940. PER.C6-PER.C6TM has been found to be more stable, particularly in handling, than, for instance, transformed human 293 cells immortalized by the adenoviral E1 region. PER.C6-PER.C6TM cells have been extensively characterized and documented, demonstrating good process of upscaling, suspension growth and growth factor independence. Furthermore, PER.C6-PER.C6TM can be incorporated into a suspension in a highly reproducible manner, making it particularly suitable for large-scale production. In this regard, the PER.C6-PER.C6TM cell line has been characterized for bioreactor growth, where it can grow to very high densities.

[0077] The cells of the present invention, in particular PER.C6-PER.C6TM, can advantageously be cultured in the absence of animal- or human-derived serum, or animal- or human-derived serum components. Thus, isolation of monoclonal antibodies is simplified and safety is enhanced due to the absence of additional human or animal proteins in the culture. The absence of serum further increases reliability of the system since use of synthetic media, as

contemplated herein, enhances reproducibility.

[0078] The invention further provides the use of a recombinant mammalian cell for the production of at least one variable domain of an immunoglobulin, the cell having a sequence encoding at least an immortalizing E1 protein of an adenovirus or a functional derivative, homologue or fragment thereof in its genome, which cell does not produce structural adenoviral proteins. In another embodiment, the invention provides such a use wherein the cell is derived from a primary cell, preferably wherein the human cell is a PER.C6-PER.C6TM cell or a derivative thereof.

Please amend paragraphs [0080] and [0081] as follows:

[0080] In another embodiment, the invention provides a human cell having a sequence encoding E1 of an adenovirus (or a functional derivative, homologue or fragment thereof) in its genome, which cell does not produce structural adenoviral proteins, and having a gene encoding a human recombinant protein, preferably a human cell which is derived from PER.C6-PER.C6TM as deposited under ECACC no. 96022940.

[0081] In yet another embodiment, the invention provides such a human cell, PER.C6-PER.C6TM/E2A, which further includes a sequence encoding E2A (or a functional derivative, analogue or fragment thereof) in its genome, preferably wherein the E2A is temperature sensitive.

Please amend paragraph [0086] as follows:

[0086] In cells according to the invention, it is important that the E1 gene sequences are not lost during the cell cycle. It is therefore preferred that the sequence encoding at least one gene product of the E1 gene is present in the genome of the (human) cell. For reasons of safety, care is best taken to avoid unnecessary adenoviral sequences in the cells according to the invention. It is thus another embodiment of the invention to provide cells that do not produce adenoviral structural proteins. However, in order to achieve large-scale (continuous) virus protein production through cell culture, it is preferred to have cells capable of growing without needing anchorage. The cells of the present invention have that capability. To have a clean and

safe production system from which it is easy to recover and, if desirable, to purify the virus protein, it is preferred to have a method according to the invention, wherein the human cell includes no other adenoviral sequences. The most preferred cell for the methods and uses of the invention is PER.C6-PER.C6™ as deposited under ECACC no. 96022940, or a derivative thereof.

Please amend paragraph [0089] as follows:

[0089] The invention also includes a method wherein the human cell can be cultured in the absence of serum. The cells according to the invention, in particular PER.C6PER.C6™, has the additional advantage that it can be cultured in the absence of serum or serum components. Thus, isolation is easy, safety is enhanced and reliability of the system is good (synthetic media are the best in reproducibility). The human cells of the invention, and in particular those based on primary cells and particularly the ones based on HER cells, are capable of normal post and peri-translational modifications and assembly. This means that they are very suitable for preparing viral proteins for use in vaccines.

Please amend paragraph [0095] as follows:

[0095] To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention. The human erythropoietin (EPO) molecule contains four carbohydrate chains. Three contain N-linkages to asparagines, and one contains an O-linkage to a serine residue. The importance of glycosylation in the biological activity of EPO has been well documented (Delorme et al. 1992; Yamaguchi et al. 1991). The cDNA encoding human EPO was cloned and expressed in PER.C6-PER.C6™ cells and PER.C6/E2A-PER.C6™/E2A cells, expression was shown, and the glycosylation pattern was analyzed.

Please amend paragraph [0098] as follows:

[0098] Plasmid pcDNA2000/Hyg(-) was digested with PmlI, and the linearized plasmid lacking the Hygromycin resistance marker gene was purified from gel and religated. The resulting plasmid was designated pcDNA2000. Plasmid pcDNA2000 was digested with PmlI

and dephosphorylated by SAP at both termini. Plasmid pIG-GC9 containing the wild type human DHFR cDNA (Havenga et al. 1998) was used to obtain the wild type DHFR-gene by polymerase chain reaction (PCR) with introduced, noncoding PmlI sites upstream and downstream of the cDNA. PCR primers that were used were DHFR up: 5'-GAT CCA CGT GAG ATC TCC ACC ATG GTT GGT TCG CTA AAC TG-3' (SEQ ID NO: 1) and DHFR down: 5'-GAT CCA CGT GAG ATC TTT AAT CAT TCT TCT CAT ATAC-3' (SEQ ID NO: 2). The PCR-product was digested with PmlI and used for ligation into pcDNA2000 (digested with PmlI, and dephosphorylated by SAP) to obtain pcDNA2000/DHFRwt (FIG. 1). Wild type sequences and correctly used cloning sites were confirmed by double stranded sequencing. Moreover, a mutant version of the human DHFR gene (DHFRm) was used to reach a 10,000 fold higher resistance to methotrexate in PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A by selection of a possible integration of the transgene in a genomic region with high transcriptional activity. This mutant carries an amino acid substitution in position 32 (phenylalanine to serine) and position 159 (leucine to proline) introduced by the PCR procedure. PCR on plasmid pIG-GC12 (Havenga et al. 1998) was used to obtain the mutant version of human DHFR. Cloning of this mutant is comparable to wild type DHFR. The plasmid obtained with mutant DHFR was designated pcDNA2000/DHFRm.

Please amend paragraph [0104] as follows:

[0104] The plasmid pMLPI.TK (described in International Patent Application No. WO 97/00326) is an example of an adapter plasmid designed for use in combination with improved packaging cell lines like PER.C6-PER.C6™ (described in International Patent Application No. WO 97/00326 and US Patent Application Serial No. 08/892,873). First, a PCR fragment was generated from pZipDMo+PyF101(N-) template DNA (described in International Patent Publication No. PCT/NL96/00195) with the following primers: LTR-1 (5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' (SEQ ID NO: 7)) and LTR-2 (5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3' (SEQ ID NO: 8)). The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al. 1991), that was digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1

up to bp 454 followed by a promoter consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al. 1990, using the following primers: HSA1 (5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' (SEQ ID NO: 9)) and HSA2 (5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3' (SEQ ID NO: 10)). The 269 bp PCR fragment was subcloned in a shuttle vector using NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication, was then excised as a NcoI/SalI fragment and cloned into a 3.5 kb NcoI/BstBI cut pLTR10, resulting in pLTR-HSA10. This plasmid was digested with EcoRI and BamHI, after which the fragment, containing the left ITR, the packaging signal, the L420 promoter and the HSA gene, was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences, resulting in the new adapter plasmid pAd5/L420-HSA.

Please amend paragraph [0132] as follows:

[0132] PER.C6 PER.C6™ and PER.C6/E2A PER.C6™/E2A were seeded in different densities. The starting concentration of methotrexate (MTX) in these sensitivity studies ranged between 0 nM and 2500 nM. The concentration which was just lethal for both cell lines was determined; when cells were seeded in densities of 100,000 cells per well in a 6-well dish, wells were still 100% confluent at 10nM and approximately 90-100% confluent at 25nM, while most cells were killed at a concentration of 50nM and above after 6 days to 15 days of incubation. These results are summarized in table 1. PER.C6 PER.C6™ cells were tested for their resistance to a combination of Hygromycin and G418 to select outgrowing stable colonies that expressed both heavy and light chains for the respective recombinant monoclonal antibodies encoded by plasmids carrying either a hygromycin or a neomycin resistance gene. When cells were grown on normal medium containing 100ug/ml hygromycin and 250 ug/ml G418, non-transfected cells were killed and stable colonies could appear. (See, Example 7).

Please amend paragraph [0135] as follows:

[0135] Cells of cell lines ~~PER.C6-PER.C6™~~ and ~~PER.C6/E2A-PER.C6™/E2A~~ were seeded in 40 tissue culture dishes (10 cm diameter) with approximately 2-3 million cells/dish and were kept overnight under their respective conditions (10% CO₂ concentration and temperature, which is 39°C for ~~PER.C6/E2A-PER.C6™/E2A~~ and 37°C for ~~PER.C6-PER.C6™~~). The next day, transfections were all performed at 37°C using Lipofectamine (Gibco). After replacement with fresh (DMEM) medium after 4 hours, ~~PER.C6/E2A-PER.C6™/E2A~~ cells were transferred to 39°C again, while ~~PER.C6-PER.C6™~~ cells were kept at 37°C. Twenty dishes of each cell line were transfected with 5 ug Scal digested pEPO2000/DHFRwt and twenty dishes were transfected with 5 µg Scal digested pEPO2000/DHFRm, all according to standard protocols. Another 13 dishes served as negative controls for methotrexate killing and transfection efficiency, which was approximately 50%. On the next day, MTX was added to the dishes in concentrations ranging between 100 and 1000 nM for DHFRwt and 50,000 and 500,000 nM for DHFRm dissolved in medium containing dialyzed FBS. Cells were incubated over a period of 4-5 weeks. Tissue medium (including MTX) was refreshed every two-three days. Cells were monitored daily for death, comparing between positive and negative controls. Outgrowing colonies were picked and subcultured. No positive clones could be subcultured from the transfectants that received the mutant DHFR gene, most likely due to toxic effects of the high concentrations of MTX that were applied. From the ~~PER.C6-PER.C6™~~ and ~~PER.C6/E2A-PER.C6™/E2A~~ cells that were transfected with the wild type DHFR gene, only cell lines could be established in the first passages when cells were grown on 100nM MTX, although colonies appeared on dishes with 250 and 500 nM MTX. These clones were not viable during subculturing, and were discarded.

Please amend paragraph [0138] as follows:

[0138] The following selection of good producer clones was based on high expression, culturing behaviour and viability. To allow checks for long-term viability, suspension growth in roller bottles and bioreactor during extended time periods, more vials of the best producer clones were frozen, and the following best producers of each cell line were selected for further

investigations P8, P9, E17 and E55 in which "P" stands for ~~PER.C6~~PER.C6™ and "E" stands for ~~PER.C6~~PER.C6™/E2A. These clones are subcultured and subjected to increasing doses of methotrexate in a time span of two months. The concentration starts at the threshold concentration and increases to approximately 0.2 mM. During these two months, EPO ELISA experiments are performed on a regular basis to detect an increase in EPO production. At the highest methotrexate concentration, the best stable producer is selected and compared to the amounts from the best CHO clone and used for cell banking (RL). From every other clone, 5 vials are frozen. The number of amplified EPO cDNA copies is detected by Southern blotting.

Please amend paragraph [0140] as follows:

[0140] The best performing EPO producing transfected stable cell line of ~~PER.C6~~PER.C6™, P9, was brought into suspension and scaled up to 1 to 2 liter fermentors. To get P9 into suspension, attached cells were washed with PBS and subsequently incubated with JRH ExCell 525 medium for ~~PER.C6~~PER.C6™ (JRH Biosciences), after which the cells loosen from the flask and form the suspension culture. Cells were kept at two concentrations of MTX: 0 nM and 100 nM. General production levels of EPO that were reached at these concentrations (in roller bottles) were respectively 1500 and 5700 units per million seeded cells per day. Although the lower yields in the absence of MTX can be explained by removal of the integrated DNA, it seems as if there is a shut-down effect of the integrated DNA since cells that are kept at lower concentrations of MTX for longer periods of time are able to reach their former yields when they are transferred to 100 nM MTX concentrations again. (See, Example 11).

Please amend paragraph [0147] as follows:

[0147] Fresh P9 suspension cells were grown in the presence of 100 nM MTX in roller bottles and used for inoculation of 4 x 1 liter bioreactors to a density of 0.3 million cells per ml in JRH ExCell 525 medium. EPO yields were determined after 3, 5 and 7 days. The first settings that were tested were: 0.5%, 10%, 150% and as a positive control 50% Dissolved Oxygen (%DO). 50% DO is the condition in which ~~PER.C6~~PER.C6™ and P9 cells are normally kept. In another run, P9 cells were inoculated and tested for EPO production at different temperatures

(32°C, 34°C, 37°C and 39°C) in which 37°C is the normal setting for PER.C6-PER.C6TM and P9, and in the third run, fresh P9 cells were inoculated and tested for EPO production at different pH settings (pH 6.5, pH 6.8, pH 7.0 and pH 7.3). PER.C6-PER.C6TM cells are normally kept at pH 7.3. An overview of the EPO yields (3 days after seeding) is shown in FIG. 17. Apparently, EPO concentrations increase when the temperature is rising from 32 to 39°C as was also seen with PER.C6/E2A-PER.C6TM/E2A cells grown at 39°C (Table 4), and 50% DO is optimal for P9 in the range that was tested here. At pH 6.5, cells cannot survive since the viability in this bioreactor dropped beneath 80% after 7 days. EPO samples produced in these settings are checked for glycosylation and charge in 2D electrophoresis. (See, also Example 17).

Please amend paragraph [0149] as follows:

[0149] A number of cell lines described in Example 8 were used in an amplification experiment to determine the possibility of increasing the number of DHFR genes by increasing the concentration of MTX in a time span of more than two months. The concentration started at the threshold concentration (100 nM) and increased to 1800 nM with in-between steps of 200 nM, 400 nM, 800 nM and 1200 nM. During this period, EPO ELISA experiments were performed on a regular basis to detect the units per million seeded cells per day (FIG. 18). At the highest MTX concentration (1800 nM), some vials were frozen. Cell pellets were obtained and DNA was extracted and subsequently digested with BglIII, since this enzyme cuts around the wild type DHFR gene in pEPO2000/DHFRwt (FIG. 5), so a distinct DHFR band of that size would be distinguishable from the endogenous DHFR bands in a Southern blot. This DNA was run and blotted and the blot was hybridized with a radioactive DHFR probe and subsequently with an adenovirus E1 probe as a background control (FIG. 19). The intensities of the hybridizing bands were measured in a phosphorimager and corrected for background levels. These results are shown in table 3. Apparently, it is possible to obtain amplification of the DHFR gene in PER.C6-PER.C6TM cells, albeit in this case only with the endogenous DHFR and not with the integrated vector.

Please amend paragraphs [0153] through [0158] as follows:

[0153] pEPO2000/DHFRwt, pEPO2000/DHFRm and pAdApt.EPO plasmids from Example 2 are purified from *E. coli* over columns, and are transfected using lipofectamine, electroporation, PEI or other methods. PER.C6-PER.C6TM or PER.C6/E2A-PER.C6TM/E2A cells are counted and seeded in DMEM plus serum or JRH ExCell 525 medium or the appropriate medium for transfection in suspension. Transfection is performed at 37°C up to 16 hours, depending on the transfection method used, according to procedures known by a person skilled in the art. Subsequently, the cells are placed at different temperatures and the medium is replaced by fresh medium with or without serum. In the case when it is necessary to obtain medium that completely lacks serum components, the fresh medium lacking serum is removed again after 3 hours and replaced again by medium lacking serum components. For determination of recombinant EPO production, samples are taken at different time points. Yields of recombinant protein are determined using an ELISA kit (R&D Systems) in which 1 Unit equals approximately 10 ng of recombinant CHO- produced EPO protein (100,000 Units/mg). The cells used in these experiments grow at different rates, due to their origin, characteristics and temperature. Therefore, the amount of recombinant EPO produced is generally calculated in ELISA units/10⁶ seeded cells/day, taking into account that the antisera used in the ELISA kit do not discriminate between non- and highly glycosylated recombinant EPO. Generally, samples for these calculations are taken at day 4 after replacing the medium upon transfection.

[0154] PER.C6/E2A-PER.C6TM/E2A cells, transfected at 37°C using lipofectamine and subsequently grown at 39°C in the presence of serum, typically produced 3100 units/10⁶ cells/day. In the absence of serum components without any refreshment of medium lacking serum, these lipofectamine-transfected cells typically produced 2600 units/10⁶ cells/day. PER.C6-PER.C6TM cells, transfected at 37°C using lipofectamine and subsequently grown at 37°C in the presence of serum, typically produced 750 units/10⁶ cells/day and, in the absence of serum, 590 units/ 10⁶ cells/day. For comparison, the same expression plasmids pEPO2000/DHFRwt and pEPO2000/DHFRm were also applied to transfect CHO cells (ECACC deposit no. 85050302) using lipofectamine, PEI, calcium phosphate procedures and other methods. When CHO cells were transfected using lipofectamine and subsequently cultured in Hams F12 medium in the presence of serum, a yield of 190 units/10⁶ cells/day was obtained. In the absence of serum, 90 units/10⁶ cells/day were produced, although higher yields can be obtained when transfections are

being performed in DMEM.

[0155] Different plates containing attached PER.C6/E2A-PER.C6TM/E2A cells were also transfected at 37°C with pEPO2000/DHFRwt plasmid and subsequently placed at 32°C, 34°C, 37°C or 39°C to determine the influence of temperature on recombinant EPO production. A temperature-dependent production level was observed ranging from 250 to 610 units/106 seeded cells/day, calculated from a day 4 sample, suggesting that the difference between production levels observed in PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A is partly due to incubation temperatures (See, also FIG. 17). Since PER.C6/E2A-PER.C6TM/E2A grows well at 37°C, further studies were performed at 37°C.

[0156] Different plates containing attached PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A cells were transfected with pEPO2000/DHFRwt, pEPO2000/DHFRm and pAdApt.EPO using lipofectamine. Four hours after transfection, the DMEM was replaced with either DMEM plus serum or JRH medium lacking serum and EPO was allowed to accumulate in the supernatant for several days to determine the concentrations that are produced in the different mediums. PER.C6-PER.C6TM cells were incubated at 37°C, while PER.C6/E2A-PER.C6TM/E2A cells were kept at 39°C. Data from the different plasmids were averaged since they contain a similar expression cassette. Calculated from a day 6 sample, the following data were obtained: PER.C6-PER.C6TM grown in DMEM produced 400 units/106 seeded cells/day, and when they were kept in JRH medium, they produced 300 units/106 seeded cells/day. PER.C6/E2A-PER.C6TM/E2A grown in DMEM produced 1800 units/106 seeded cells/day, and when they were kept in JRH, they produced 1100 units/106 seeded cells/day. Again, a clear difference was observed in production levels between PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A, although this might partly be due to temperature differences (See, above paragraph [0140]). There was, however, a significant difference with PER.C6/E2A-PER.C6TM/E2A cells between the concentration in DMEM vs. the concentration in JRH medium, although this effect was almost completely lost in PER.C6-PER.C6TM cells.

[0157] EPO expression data obtained in this system are summarized in Table 4. PER.C6-PER.C6TM and derivatives thereof can be used for scaling up the DNA transfections system. According to Wurm and Bernard (1999), transfections on suspension cells can be performed at 1-10 liter set-ups in which yields of 1-10 mg/l (0.1-1 pg/cell/day) of recombinant

protein have been obtained using electroporation. A need exists for a system in which this can be well controlled and yields might be higher, especially for screening of large numbers of proteins and toxic proteins that cannot be produced in a stable setting. With the lipofectamine transfections on the best PER.C6-PER.C6TM cells in the absence of serum, we reached 590 units/million cells/day (+/-5.9 pg/cell/day when 1 ELISA unit is approximately 10 ng EPO), while PER.C6/E2A-PER.C6TM/E2A reached 31 pg/cell/day (in the presence of serum). The medium used for suspension cultures of PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A (JRH ExCell 525) does not support efficient transient DNA transfections using components like PEI. Therefore, the medium is adjusted to enable production of recombinant EPO after transfection of pEPO2000/DHFRwt and pEPO2000/DHFRm containing a recombinant human EPO cDNA, and pcDNA2000/DHFRwt containing other cDNA's encoding recombinant proteins.

[0158] 1 to 10 liter suspension cultures of PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A growing in adjusted medium to support transient DNA transfections using purified plasmid DNA are used for electroporation or other methods, performing transfection with the same expression plasmids. After several hours, the transfection medium is removed and replaced by fresh medium without serum. The recombinant protein is allowed to accumulate in the supernatant for several days, after which the supernatant is harvested and all the cells are removed. The supernatant is used for down stream processing to purify the recombinant protein.

Please amend paragraphs [0161] through [0163] as follows:

[0161] Example 14: Infection of attached and suspension PER.C6-PER.C6TM cells with IG.Ad5/AdApt.EPO.dE2A.

[0162] Purified viruses from Example 13 were used for transient expression of recombinant EPO in PER.C6-PER.C6TM cells and derivatives thereof. IG.Ad5/AdApt.EPO.dE2A virus was used to infect PER.C6-PER.C6TM cells, while IG.Ad5/AdApt.EPO.tetOE4 and IG.Ad5/AdApt.EPO.dE2A.tetOE4 viruses can be used to infect PER.C6/E2A-PER.C6TM/E2A cells to lower the possibility of replication and, moreover, to prevent inhibition of recombinant protein production due to replication processes. Infections

were performed on attached cells as well as on suspension cells in their appropriate medium using ranges of multiplicities of infection (moi's): 20, 200, 2000, 20000 vp/cell. Infections were performed for 4 hours in different settings ranging from 6-well plates to roller bottles, after which the virus containing supernatant was removed. The cells were washed with PBS or directly refreshed with new medium. Then, cells were allowed to produce recombinant EPO for several days, during which samples were taken and EPO yields were determined. Also, the number of viable cells compared to dead cells was checked. The amount of EPO that was produced was again calculated as ELISA unit seeded cells/day, because the different cell lines have different growth characteristics due to their passage number and environmental circumstances such as temperature and selective pressures. Suspension growing PER.C6 PER.C6TM cells were seeded in 250 ml JRH ExCell 525 medium in roller bottles (1 million cells per ml) and subsequently infected with purified IG.Ad5/AdApt.EPO.dE2A virus with an moi of 200 vp/cell. The estimation used for vp determination was high (vp/IU ratio of this batch is 330, which indicates an moi of 0.61 IU's/cell). Thus, not all cells were hit by an infectious virus. A typical production of recombinant EPO in this setting from a day 6 sample was 190 units/10⁶ seeded cells/day, while in a setting in which 50% of the medium including viable and dead cells was replaced by fresh medium, approximately 240 units/10⁶ cells/day were obtained. The refreshment did not influence the viability of the viable cells, but the removed recombinant protein could be added to the amount that was obtained at the end of the experiment, albeit present in a larger volume. An identical experiment was performed with the exception that cells were infected with an moi of 20 vp/cell, resembling approximately 0.06 Infectious Units/cell. Without refreshment, a yield of 70 ELISA units/10⁶ cells/day was obtained, while in the experiment in which 50% of the medium was refreshed at day 3, a typical amount of 80 units/10⁶ cells/day was measured. This indicates that there is a dose response effect when an increasing number of infectious units is used for infection of PER.C6 PER.C6TM cells.

[0163] Furthermore, PER.C6 PER.C6TM cells growing in DMEM were seeded in 6-well plates and left overnight in 2 ml DMEM with serum to attach. The next day, cells were infected with another batch of IG.Ad5/AdApt.EPO.dE2A virus (vp/IU ratio 560) with an moi of 200 vp/cells (0.35 Infectious Units/cell). After 4 hours, the virus containing medium was removed and replaced by fresh medium including serum, and cells were left to produce recombinant EPO

for more than two weeks with replacement of the medium with fresh medium every day. The yield of recombinant EPO production calculated from a day 4 sample was 60 units/106 cells/day.

Please amend paragraphs [0165] and [0166] as follows:

[0165] Due to the fact that a tTA-tetO regulated expression of the Early region 4 of adenovirus (E4) impairs the replication capacity of the recombinant virus in the absence of active E4, it is also possible to use the possible protein production potential of the PER.C6/E2A PER.C6TM/E2A as well as its parental cell line PER.C6-PER.C6TM to produce recombinant proteins in a setting in which a recombinant adenovirus is carrying the human EPO cDNA as the transgene and in which the E4 gene is under the control of a tet operon. Then, very low levels of E4 mRNA are being produced, resulting in very low but detectable levels of recombinant and replicating virus in the cell line PER.C6/E2A-PER.C6TM/E2A and no detectable levels of this virus in PER.C6-PER.C6TM cells. To produce recombinant EPO in this way, the two viruses IG.Ad5/AdApt.EPO.tetOE4 and IG.Ad5/AdApt.EPO.dE2A.tetOE4 are used to infect PER.C6 PER.C6TM cells and derivatives thereof. Attached and suspension cells are infected with different moi's of the purified adenoviruses in small settings (6-well plates and T25 flasks) and large settings (roller bottles and fermentors). Samples are taken at different timepoints and EPO levels are determined.

[0166] Since viruses that are deleted in E1 and E2A in the viral backbone can be complemented in PER.C6/E2A-PER.C6TM/E2A cells but not in the original PER.C6-PER.C6TM cells, settings are used in which a mixture of both cell lines is cultured in the presence of IG.Ad5/AdApt.EPO.dE2A virus. The virus will replicate in PER.C6TM/E2A, followed by lysis of the infected cells and a subsequent infection of PER.C6-PER.C6TM or PER.C6/E2A PER.C6TM/E2A cells. In contrast, in PER.C6-PER.C6TM cells, the virus will not replicate and the cells will not lyse due to viral particle production, but will produce recombinant EPO that will be secreted in the supernatant. A steady state culture/replication/EPO production system is set up in which fresh medium and fresh PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A cells are added at a constant flow, while used medium, dead cells and debris are removed. Together with this, recombinant EPO is taken from the system and used for purification in a down stream processing procedure in which virus particles are removed.

Please amend paragraphs [0168] through [0174] as follows:

[0168] Large batches of growing cells are produced in bioreactors; the secreted recombinant human EPO protein is purified according to procedures known by one of skill in the art. The purified recombinant human EPO protein from PER.C6-PER.C6™ and PER.C6/E2A PER.C6™/E2A stable clones or transfectants is checked for glycosylation and folding by comparison with commercially available EPO and EPO purified from human origin (urine) using methods known to one of skill in the art (See, Examples 16 and 17). Purified and glycosylated EPO proteins from PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A are tested for biological activity in *in vitro* experiments and in mouse spleens as described (Krystal (1983) and *in vitro* assays (See, Example18).

[0169] Example 16: Activity of beta-galactoside alpha 2,6-sialyltransferase in PER.C6PER.C6™.

[0170] It is known that CHO cells do not contain a gene for beta-galactoside alpha 2,6-sialyltransferase, resulting in the absence of alpha 2,6-linked sialic acids at the terminal ends of – and O-linked oligosaccharides of endogenous and recombinant glycoproteins produced on these CHO cells. Since the alpha 2,3-sialyltransferase gene is present in CHO cells, proteins that are produced on these cells are typically from the 2,3 linkage type. EPO that was purified from human urine does, however, contain both alpha 2,3- and alpha 2,6-linked sialic acids. To determine whether PER.C6-PER.C6™ cells, being a human cell line, are able to produce recombinant EPO containing both alpha 2,3- and alpha 2,6-linkages, a direct neuraminidase assay was performed on recombinant EPO produced on PER.C6-PER.C6™ cells after transfection with EPO expression vectors. As a control, commercially available Eprex samples were used, which were derived from CHO cells and which should only contain sialic acid linkages of the alpha 2,3 type. The neuraminidases that were used were from Newcastle Disease Virus (NDV) that specifically cleaves alpha 2,3-linked neuraminic acids (sialic acids) from N– and O-linked glycans, and from Vibro cholerae (VC) that non-specifically cleaves all terminal N– or O-linked sialic acids (alpha 2,3, alpha 2,6 and alpha 2,8 linkages). Both neuraminidases were from Boehringer and were incubated with the samples according to guidelines provided by

the manufacturer. Results are shown in FIG. 21A. In lanes 2 and 3 (treatment with NDV neuraminidase), a slight shift is observed as compared to lane 1 (non-treated PER.C6-PER.C6TM EPO). When this EPO sample was incubated with VC derived neuraminidase, an even faster migrating band is observed as compared to NDV treated samples. However, with the commercially available Eprex, only a shift was observed when NDV derived neuraminidase was applied (lanes 6 and 7 compared to the non-treated sample in lane 5) and not when VC neuraminidase was used (lane 8).

[0171] To definitely establish that no sialic acids of the alpha 2,6 linkage type are present on CHO cells, but that they do exist in proteins present on the cell surface of PER.C6 PER.C6TM cells, the following experiment was performed: CHO cells were released from the solid support using trypsin-EDTA, while for PER.C6PER.C6TM, suspension cells were used. Both suspensions were washed once with Mem-5% FBS and incubated in this medium for 1 h at 37° C. After washing with PBS, the cells were resuspended to approximately 106 cells/ml in binding medium (Tris-buffered saline, pH 7.5, 0.5%BSA, and 1mM each of MgCl₂, MnCl₂ and CaCl₂). Aliquots of the cells were incubated for 1 h at room temperature with DIG-labeled lectins, *Sambucus nigra* agglutinin ("SNA") and *Maackia amurensis* agglutinin ("MAA"), which specifically bind to sialic acid linkages of the alpha 2,6 Gal and alpha 2,3 Gal types, respectively. Control cells were incubated without lectins. After 1 hour, both lectin-treated and control cells were washed with PBS and then incubated for 1 hour at room temperature with FITC-labeled anti-DIG antibody (Boehringer-Mannheim). Subsequently, the cells were washed with PBS and analyzed for fluorescence intensity on a FACsort apparatus (Becton Dickinson). The FACS analysis is shown in FIG. 21B. When the SNA lectin is incubated with CHO cells, no shift is seen as compared to non-treated cells, while when this lectin is incubated with PER.C6 PER.C6TM cells, a clear shift (dark fields) is observed as compared to non-treated cells (open fields). When both cell lines are incubated with the MAA lectin, both cell lines give a clear shift as compared to non-treated cells.

[0172] From these EPO digestions and FACS results, it is concluded that there is a beta-galactoside alpha 2,6 sialyltransferase activity present in human PER.C6-PER.C6TM cells which is absent in CHO cells.

[0173] Example 17: Determination of sialic acid content in PER.C6-PER.C6TM

produced EPO.

[0174] The terminal neuraminic acids (or sialic acids) that are present on the – and O-linked glycans of EPO protect the protein from clearance from the bloodstream by enzymes in the liver. Moreover, since these sialic acids are negatively charged, one can distinguish between different EPO forms depending on their charge or specific pI. therefore, EPO produced on PER.C6-PER.C6™ and CHO cells was used in 2-dimensional electrophoresis in which the first dimension separates the protein on charge (pH range 3-10) and the second dimension separates the proteins further on molecular weight. Subsequently, the proteins were blotted and detected in a western blot with an anti-EPO antibody.

Please amend paragraphs [0176] and [0177] as follows:

[0176] In FIG. 22A, a number of EPO samples are shown that were derived from P9 supernatants. P9 is the PER.C6-PER.C6™ cell line that stably expresses recombinant human EPO (See, Example 8). These samples were compared to commercially available Eprex, which contains only EPO forms harboring approximately 9 to 14 sialic acids. Eprex should, therefore, be negatively charged and be focusing towards the pH 3 side of the gel. FIG. 22B shows a comparison between EPO derived from P9 in an attached setting in which the cells were cultured on DMEM medium and EPO derived from CHO cells that were transiently transfected with the pEPO2000/DHFRwt vector. Apparently, the lower forms of EPO cannot be detected in the CHO samples, whereas all forms can be seen in the P9 sample. The sialic acid content is given by numbering the bands that were separated in the first dimension from 1 to 14. It is not possible to determine the percentage of each form of EPO molecules present in the mixtures because the western blot was performed using ECL, and because it is unknown whether glycosylation of the EPO molecule or transfer of the EPO molecule to the nitrocellulose inhibits recognition of the EPO molecule by the antibody. However, it is possible to determine the presence of the separate forms of sialic acid containing EPO molecules. It can be concluded that PER.C6-PER.C6™ is able to produce the entire range of 14 sialic acid containing isoforms of recombinant human EPO.

[0177] Example 18: *in vitro* functionality of PER.C6-PER.C6™ produced EPO.

Please amend paragraphs [0180] and [0181] as follows:

[0180] EPO produced on ~~PER.C6/E2A-PER.C6TM/E2A~~ cells was used to stimulate TF-1 cells as follows: Cells were seeded in 96-well plates with a density of around 10,000 cells per well in medium lacking IL3 or GM-CSF, which are the growth factors that can stimulate indefinite growth of these cells in culture. Subsequently, medium is added, resulting in final concentrations of 0.0001, 0.001, 0.01, 0.1, 1 and 10 units per ml. These units were determined by ELISA, while the units of the positive control Eprex were known (4000 units per ml) and were diluted to the same concentration. Cells were incubated with these EPO samples for 4 days, after which an MTS assay (Promega) was performed to check for viable cells by fluorescence measurement at 490 nm (fluorescence is detectable after transfer of MTS into formazan). FIG. 23 shows the activity of two samples derived from ~~PER.C6/E2A-PER.C6TM/E2A~~ cells that were transfected with an EPO expression vector and subsequently incubated at 37°C and 39°C for 4 days. The results suggest that samples obtained at 39°C are more active than samples obtained at 37°C, which might indicate that the sialic acid content is suboptimal at higher temperatures. It is hereby shown that ~~PER.C6-PER.C6TM~~ produced EPO can stimulate TF-1 cells in an *in vitro* assay, strongly suggesting that the EPO that is produced on this human cell line can interact with the EPO receptor and stimulate differentiation.

[0181] Example 19: Production of recombinant murine, humanized and human monoclonal antibodies in ~~PER.C6-PER.C6TM~~ and ~~PER.C6/E2A-PER.C6TM/E2A~~.

Please amend paragraph [0184] as follows:

[0184] Plasmids containing the cDNA's of the heavy and light chain of a murine and a humanized monoclonal antibody are transfected and, after several days, the concentration of correctly folded antibody is determined using methods known to persons skilled in the art. Conditions such as temperature and used medium are checked for both ~~PER.C6-PER.C6TM~~ and ~~PER.C6/E2A-PER.C6TM/E2A~~ cells. Functionality of the produced recombinant antibody is controlled by determination of affinity for the specified antigen.

Please amend paragraph [0186] as follows:

[0186] cDNA's encoding a heavy and a light chain are cloned in two different systems: one in which the heavy and light chains are integrated into one single adapter plasmid (a modified pAdApt.pac) and the other in which heavy and light chain cDNA's are cloned separately into two different adapters (each separately in pAdApt.pac). In the first system, viruses are propagated that carry an E1 deletion (dE1) together with a E2A deletion (dE2A) or both deletions in the context of a tetOE4 insertion in the adenoviral backbone. In the second system, the heavy and light chains are cloned separately in pAdApt.pac and separately propagated to viruses with the same adenoviral backbone. These viruses are used to perform single or co-infections on attached and suspension growing PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A cells. After several days, samples are taken to determine the concentration of full length recombinant antibodies, after which the functionality of these antibodies is determined using the specified antigen in affinity studies.

Please amend paragraph [0188] as follows:

[0188] Expression plasmids carrying the heavy and light chain together and plasmids carrying the heavy and light chain separately are used to transfect attached PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A and CHO-dhfr cells. Subsequently, cells are exposed to MTX and/or hygromycin and neomycin to select for integration of the different plasmids. Moreover, a double selection with G418 and hygromycin is performed to select for integration of plasmids that carry the neomycin and hygromycin resistance gene. Expression of functional full length monoclonal antibodies is determined and best expressing clones are used for subsequent studies including stability of integration, copy number detection, determination of levels of both subunits and ability to amplify upon increase of MTX concentration after the best performing cell lines are used for mAb production in larger settings such as perfused and (fed-) batch bioreactors, after which optimization of quantity and quality of the mAbs is executed.

Please amend paragraph [0190] as follows:

[0190] PER.C6-PER.C6™ cells were seeded in DMEM plus 10% FBS in 47 tissue

culture dishes (10 cm diameter) with approximately 2.5 x 10⁶ cells per dish and were kept overnight under their normal culture conditions (10% CO₂ concentration and 37°C). The next day, co-transfections were performed in 39 dishes at 37°C using Lipofectamine in standard protocols with 1 ug MunI digested and purified pUBS-Heavy2000/Hyg(-) and 1 ug ScaI digested and purified pUBS-Light2001/Neo (See, Example 3) per dish, while 2 dishes were co-transfected as controls with 1 ug MunI digested and purified pcDNA2000/Hyg(-) and 1 ug ScaI digested and purified pcDNA2001/Neo. As a control for transfection efficiency, 4 dishes were transfected with a LacZ control vector, while 2 dishes were not transfected and served as negative controls.

Please amend paragraphs [0194] through [0196] as follows:

[0194] Transfections as described for pUBS-Heavy2000/Hyg(-) and pUBS-Light2001/Neo are performed with pUBS2-Heavy2000/Hyg(-) and pUBS2-Light2001/Neo in PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A and selection is performed with either subsequent incubation with hygromycin followed by G418 or as described above with a combination of both selection reagents. CHO-dhfr cells are transfected with pUBS2-Heavy2000/Hyg(-) and pUBS2-Light2001/DHFRwt as described herein and selection is performed in a sequential way in which cells are first selected with hygromycin, after which an integration of the light chain vector is controlled by selection on MTX.

[0195] Furthermore, PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A cells are also used for transfections with pUBS-3000/Hyg(-) and pUBS2-3000/Hyg(-), while CHO-dhfr cells are transfected with pUBS-3000/DHFRwt and pUBS2-3000/DHFRwt, after which a selection and further amplification of the integrated plasmids are performed by increasing the MTX concentration. In the case of the pcDNAs3000 plasmids, an equal number of mRNA's of both the heavy and light chain is expected, while in the case of two separate vectors, it is unclear whether a correct equilibrium is achieved between the two subunits of the immunoglobulin.

[0196] Transfections are also being performed on PER.C6-PER.C6™, PER.C6/E2A-PER.C6™/E2A and CHO-dhfr with expression vectors described in Examples 4 and 5 to obtain stable cell lines that express the humanized IgG1 mAb CAMPATH-1H and the humanized IgG1 mAb 15C5 respectively.

Please amend paragraph [0198] as follows:

[0198] From ~~PER.C6-PER.C6™~~ cells transfected with pUBS-Heavy2000/Hyg (-) and PUBL-Light2001/Neo, approximately 300 colonies that were growing in medium containing Hygromycin and G418 were generally grown subsequently in 96-well, 24-well and 6-well plates in their respective medium plus their respective selecting agents. Cells that were able to grow in 24 well plates were checked for mAb production by using the ELISA described in Example 26. If cells scored positively, at least one vial of each clone was frozen and stored, and cells were subsequently tested and subcultured. The selection of a good producer clone is based on high expression, culturing behavior and viability. To allow checks for long term viability, amplification of the integrated plasmids and suspension growth during extended time periods, best producer clones are frozen, of which a number of the best producers of each cell line are selected for further work. Similar experiments are being performed on CHO-dhfr cells transfected with different plasmids and ~~PER.C6-PER.C6™~~ and ~~PER.C6/E2A-PER.C6™/E2A~~ cells that were transfected with other combinations of heavy and light chains and other combinations of selection markers.

Please amend paragraphs [0200] and [0201] as follows:

[0200] The best UBS-54 producing transfected cell line of ~~PER.C6-PER.C6™~~ is brought into suspension by washing the cells in PBS and then culturing the cells in JRH ExCell 525 medium, first in small culture flasks and subsequently in roller bottles, and scaled up to 1 to 2 liter fermentors. Cells are kept on hygromycin and G418 selection until it is proven that integration of the vectors is stable over longer periods of time. This is done when cells are still in their attached phase or when cells are in suspension.

[0201] Suspension growing mAb producing ~~PER.C6-PER.C6™~~ cells are generally cultured with hygromycin and G418 and used for inoculation of bioreactors from roller bottles. Production yields, functionality and quality of the produced mAb is checked after growth of the cells in perfused bioreactors and in fed batch settings.

Please amend paragraph [0205] as follows:

[0205] In an initial run, mAb producing PER.C6-PER.C6™ suspension cells that are grown on roller bottles are used to inoculate a 2 liter bioreactor in the absence of selecting agents to a density of 0.3 to 0.5 million cells per ml in a working volume of 300 to 500 ml and are left to grow until the viability of the cell culture drops to 10%. As a culture lifetime standard, it is determined at what day after inoculation the viable cell density drops beneath 0.5 million cells per ml. Cells normally grow until a density of 2 to 3 million cells per ml, after which the medium components become limiting and the viability decreases. Furthermore, it is determined how much of the essential components, such as glucose and amino acids in the medium are being consumed by the cells. Next to that, it is determined what amino acids are being produced and what other products are accumulating in the culture. Depending on this, concentrated feeding samples are being produced that are added at regular time points to increase the culture lifetime and thereby increase the concentration of the mAb in the supernatant. In another setting, 10x concentrated medium samples are developed that are added to the cells at different time points and that also increase the viability of the cells for a longer period of time, finally resulting in a higher concentration of mAb in the supernatant.

Please amend paragraph [0207] as follows:

[0207] The correct combinations of the UBS-54 heavy and light chain genes containing vectors were used in transient transfection experiments in PER.C6-PER.C6™ cells. For this, it is not important which selection marker is introduced in the plasmid backbone, because the expression lasts for a short period (2-3 days). The transfection method is generally lipofectamine, although other cationic lipid compounds for efficient transfection can be used. Transient methods are extrapolated from T25 flasks settings to at least 10-liter bioreactors. Approximately 3.5 million PER.C6-PER.C6™ and PER.C6/E2A-PER.C6™/E2A cells were seeded at day 1 in a T25 flask. At day 2, cells were transfected with, at most, 8 ug plasmid DNA using lipofectamine and refreshed after 2-4 hours and left for 2 days. Then, the supernatant was harvested and antibody titers were measured in a quantitative ELISA for human IgG1 immunoglobulins (CLB, see also Example 26). Levels of total human antibody in this system are

approximately 4.8 ug/million seeded cells for PER.C6-PER.C6TM and 11.1 ug/million seeded cells for PER.C6/E2A-PER.C6TM/E2A. To determine how much of the produced antibody is of full size and built up from two heavy and two light chains, as well as the expression levels of the heavy and/or light chain alone and connected by disulfide bridges, control ELISA's recognizing the sub-units separately are developed. Different capturing and staining antibody combinations are used that all detect human(ized) IgG1 sub-units. Supernatants of PER.C6-PER.C6TM transfectants (transfected with control vectors or pUBS-Heavy2000/Hyg(-) and pUBS-Light2001/DHFRwt) were checked for full sized mAb production (FIG. 24). Samples were treated with and without DTT, wherein one can distinguish between full sized mAb (nonreduced) and heavy and light chain separately (reduced). As expected, the heavy chain is only secreted when the light chain is co-expressed and most of the antibody is of full size.

Please amend paragraphs [0209] and [0210] as follows:

[0209] PER.C6-PER.C6TM and derivatives thereof are used for scaling up the DNA transfections system. According to Wurm and Bernard (1999), transfections on suspension cells can be performed at 1-10 liter set-ups in which yields of 1-10 mg/l (0.1-1 pg/cell/day) of recombinant protein have been obtained using electroporation.

[0210] A need exists for a system in which this can be well controlled and yields might be higher, especially for screening of large numbers of proteins and toxic proteins that cannot be produced in a stable setting. Moreover, since cell lines such as CHO are heavily affected by apoptosis-inducing agents such as lipofectamine, the art teaches that there is a need for cells that are resistant to this. Since PER.C6-PER.C6TM is hardly affected by transfection methods, it seems that PER.C6-PER.C6TM and derivatives thereof are useful for these purposes. One to 50 liter suspension cultures of PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A growing in adjusted medium to support transient DNA transfections using purified plasmid DNA are used for electroporation or other methods, performing transfection with the same expression plasmids. After several hours, the transfection medium is removed and replaced by fresh medium without serum. The recombinant protein is allowed to accumulate in the supernatant for several days, after which the supernatant is harvested and all the cells are removed. The supernatant is used for down stream processing to purify the recombinant protein.

Please amend paragraph [0212] as follows:

[0212] Heavy and light chain cDNA's of the antibodies described in Examples 3, 4 and 5 are cloned into recombinant adenoviral adapter plasmids separately and in combination. The combinations are made to ensure an equal expression level for both heavy and light chains of the antibody to be formed. When heavy and light chains are cloned separately, viruses are being produced and propagated separately, of which the infectability and the concentration of virus particles are determined and finally co-infected into PER.C6-PER.C6™ and derivatives thereof to produce recombinant mAbs in the supernatant. Production of adapter vectors, recombinant adenoviruses and mAbs is as described for recombinant EPO (See, Examples 13 and 14).

Please amend paragraph [0218] as follows:

[0218] cDNA sequences of genes encoding hemagglutinin (HA) and neuraminidase (NA) proteins of known and regularly appearing novel influenza virus strains are being determined and generated by PCR with primers for convenient cloning into pcDNA2000, pcDNA2001, pcDNA2002 and pcDNAs3000 vectors (See, Example1). Subsequently, these resulting expression vectors are being transfected into PER.C6-PER.C6™ and derivatives thereof for stable and transient expression of the recombinant proteins to result in the production of recombinant HA and NA proteins that are therefore produced in a complete standardized way with human cells under strict and well-defined conditions. Cells are allowed to accumulate these recombinant HA and NA proteins for a standard period of time. When the pcDNAs3000 vector is used, it is possible to clone both cDNA's simultaneously and have the cells produce both proteins at the same time. From separate or combined cultures, the proteins are being purified following standard techniques and final HA and NA titers are being determined and activities of the proteins are checked by persons skilled in the art. Then, the purified recombinant proteins are used for vaccination studies and finally used for large-scale vaccination purposes.

Please amend paragraphs [0221] and [0222] as follows:

[0221] Since the levels of recombinant protein production in stable and transiently

transfected and infected PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A are extremely high and since a higher expression level is usually obtained upon DHFR dependent amplification due to increase of MTX concentration, an "out-titration" of the endogenous levels of enzymes that are involved in post-translational modifications might occur.

[0222] Therefore, cDNA's encoding human enzymes involved in different kinds of post-translational modifications and processes such as glycosylation, phosphorylation, carboxylation, folding and trafficking are being overexpressed in PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A to enable a more functional recombinant product to be produced to extreme levels in small and large settings. It was shown that CHO cells can be engineered in which an alpha-2,6-sialyltransferase was introduced to enhance the expression and bioactivity of tPA and human erythropoietin (Zhang et al. 1998, Minch et al. 1995, Jenkins et al. 1998). Other genes such as beta 1,4-galactosyltransferase were also introduced into insect and CHO cells to improve the N-linked oligosaccharide branch structures and to enhance the concentration of sialic acids at the terminal residues (Weikert et al. 1999; Hollister et al. 1998). PER.C6-PER.C6TM cells are modified by integration of cDNA's encoding alpha 2,3-sialyltransferase, alpha 2,6-sialyltransferase and beta 1,4-galactosyltransferase proteins to further increase the sialic acid content of recombinant proteins produced on this human cell line.

Please amend paragraphs [0227] through [0229] as follows:

[0227] PER.C6-PER.C6TM cells and derivatives thereof do express the E1A and E1B genes of adenovirus. Other human cells, such as A549 cells, are being used to stably overexpress adenovirus E1B to determine the anti-apoptotic effects of the presence of the adenovirus E1B gene as described for CHO cells (See, Example 29). Most cells do respond to transfection agents such as lipofectamine or other cationic lipids, resulting in massive apoptosis and finally resulting in low concentrations of the recombinant proteins that are secreted, simply due to the fact that only few cells survive the treatment. Stable E1B overexpressing cells are compared to the parental cell lines in their response to overexpression of toxic proteins or apoptosis inducing proteins and their response to transfection agents such as lipofectamine.

[0228] Example 31: Generation of PER.C6-PER.C6TM derived cell lines lacking a

functional DHFR protein.

[0229] PER.C6-PER.C6™ cells are used to knock out the DHFR gene using different systems to obtain cell lines that can be used for amplification of the exogenous integrated DHFR gene that is encoded on the vectors that are described in Examples 1 to 5 or other DHFR expressing vectors. PER.C6-PER.C6™ cells are screened for the presence of the different chromosomes and are selected for a low copy number of the chromosome that carries the human DHFR gene. Subsequently, these cells are used in knock-out experiments in which the open reading frame of the DHFR gene is disrupted and replaced by a selection marker. To obtain a double knock-out cell line, both alleles are removed via homologous recombination using two different selection markers or by other systems as, for instance, described for CHO cells (Urlaub et al. 1983).

Please amend paragraph [0234] as follows:

[0234] PER.C6-PER.C6™ and derivatives thereof are being used to stably express recombinant proteins using the glutamine synthetase (GS) system. First, cells are being checked for their ability to grow in glutamine free medium. If cells cannot grow in glutamine free medium, this means that these cells do not express enough GS, finally resulting in death of the cells. The GS gene can be integrated into expression vectors as a selection marker (as is described for the DHFR gene) and can be amplified by increasing the methionine sulphoximine (MSX) concentration resulting in overexpression of the recombinant protein of interest, since the entire stably integrated vector will be co-amplified as was shown for DHFR. The GS gene expression system became feasible after a report of Sanders et al. (1984) and a comparison was made between the DHFR selection system and GS by Cockett et al. (1990). The production of recombinant mAbs using GS was first described by Bebbington et al. (1992).

Please amend paragraph [0238] as follows:

[0238] The expression vector is transfected into PER.C6-PER.C6™, derivatives thereof and CHO-dhfr cells to obtain stable producing cell lines. Differences in glycosylation between CHO-produced and PER.C6-PER.C6™ produced gp120 are being determined in 2D

electrophoresis experiments and subsequently in Mass Spectrometry experiments, since gp120 is a heavily glycosylated protein with mainly O-linked oligosaccharides. The recombinant protein is purified by persons skilled in the art and subsequently used for functionality and other assays. Purified protein is used for vaccination purposes to prevent HIV infections.

Please amend TABLE 1 as follows:

TABLE 1. Summary of methotrexate (MTX) killing of PER.C6-PER.C6TM and PER.C6/E2A-PER.C6TM/E2A after 6 and 15 days of incubation with different MTX concentrations. Cells were seeded at day 0 and incubations with MTX started at day 1 and continued for 6 days. Then, confluency (%) was scored and the medium was replaced by fresh medium plus MTX and incubation was continued for another 9 days, after which confluency (%) was scored again (day 15).

<u>PER.C6 PER.C6TM</u>		0	1	5	10	25	50	100	250	500	1000	2500	nM MTX
1E5 cells/well	day 6	70	70	70	60	<5	<1	0.5	0	0	0	0	% confluency
6-well plate	day 15	100	100	100	100	<10	<5	0	0	0	0	0	% confluency

<u>PER.C6 PER.C6TM/E2A</u>		0	1	5	10	25	50	100	250	500	1000	2500	nM MTX
1E5 cells/well	day 6	100	100	100	100	<100	5	5	4	1	<1	<1	% confluency
6-well plate	day 15	100	100	100	100	<10	<5	0	0	0	0	0	% confluency

Please amend TABLE 2 as follows:

TABLE 2. Attached ~~PER.C6-PER.C6™~~ and ~~PER.C6/E2A-PER.C6™/E2A~~ cell lines that stably express recombinant human EPO. Cell lines were generated by stable integration and expression of pEPO2000/DHFRwt (FIG. 5). Production levels were determined in the supernatant, after growth of 4 days in a T25 flask setting in the presence of 100 nM MTX.

PER.C6-PER.C6™ cell lines	ELISA units/1E6 seeded cells/day
P3	735
P5	0
P7	1733
P8	2522
P9	3839
P13	0
P15	0
P42	<1

PER.C6PER.C6™/E2A cell lines	ELISA units/1E6 seeded cells/day
E17	325
E55	1600

Please amend TABLE 4 as follows:

TABLE 4.

EPO yields in transient DNA transfections. Yields per million seeded cells were determined with an EPO ELISA on supernatants from ~~PER.C6PER.C6™~~, ~~PER.C6/E2A-PER.C6™/E2A~~ and CHO cells that were transfected with pEPO2000/DHFRwt expression vector in the absence or presence of Fetal Bovine Serum at different incubation temperatures, as described in Example 12.

Cell line	± FBS	Temp.	EPO yields (ELISA units/1E6 cells/day)
PER.C6PER.C6™/E2A	+	39 C	3100
PER.C6PER.C6™/E2A	-	39 C	2600
PER.C6PER.C6™	+	37 C	750
PER.C6PER.C6™	-	37 C	590
CHO	+	37 C	190
CHO	-	37 C	90

Please amend TABLE 5 as follows:

TABLE 5.

EPO yields obtained after viral infections. Yields per million seeded cells were determined with an EPO ELISA on supernatants from ~~PER.C6-PER.C6™~~ cells that were infected with recombinant IG.Ad5.AdApt.EPO.dE2A adenovirus as described in Example 14. Two different batches of the virus were used with different vp/IU ratios (330 and 560) in two different settings (roller bottle suspension cultures and 6-wells attached cultures).

moi yields	ratio	culture	medium	refreshment	EPO
(virus particles per cell)	(virus particles/ infectious units)	conditions			(ELISA units/ 1E6 cells/day)
200	330	roller bottle	JRH	day 3	240
200	330	roller bottle	JRH	none	190
20	330	roller bottle	JRH	day 3	80
20	330	roller bottle	JRH	none	70
200	560	6-wells	DMEM+FBS	every day	60